context of a resting nuclear perfusion scintigram employing SPECT imaging using ^{99m}Tc-sestamibi by a single blinded reader.

RESULTS

SUBJECTS ENROLLED

There were 42 subjects enrolled who were in stable condition after a 1st Q-wave (transmural) myocardial infarction; these patients were referred for nuclear perfusion studies prior to hospital discharge. The age range of those enrolled was from 39 to 83 years of age; 41 subjects received AF0150 (1 subject was not treated).

TREATMENT

Acceptable images (good technical quality and availability of views) were provided for 20 subjects for endocardial border delineation (EBD), 19 subjects for segmental wall motion (SWM), 16 subjects for ejection fraction (EF), and 20 subjects for nuclear perfusion studies. Of note are the following changes in conduct of the study:

1. Data analysis and reporting were limited to images obtained following intravenous infusion of AF0150 due to the inability to collect matched sets of images following bolus administration doses.
2. Data from the 2nd harmonic ultrasound mode was not evaluated due to the inconsistent collection of images.

According to the sponsor, efficacy results were as follows –

EBD: The number of ventricular segments visualized was increased, although the degree of visualization and differences between the non-contrast and contrast ECHOs were not significantly different.

EF: There was no statistically significant difference between non-contrast and contrast ECHO determinations.

SWM: There was poor agreement between the readers; no standard of reference was applied for this study.

SPECT: There was agreement between the contrast ECHO and SPECT (as the standard for myocardial perfusion) in 70% of the subjects.

SAFETY RESULTS

Refer to the Integrated Safety Summary write-up. A total of 46 adverse events were reported among 17 (42%) of the 41 AF0150-treated subjects, with 8 serious AE's (with 1 death due to myocardial infarction) reported among 4 subjects. None of the serious AE's were attributed to the study drug. There were 11 (27%) subjects having AE's involving the cardiovascular system; atrial fibrillation (3 subjects; 7%), hypotension (3 subjects; 7%) and hypertension (2 subjects; 5%) were the most frequently reported from that organ-system. Changes in laboratory values, echocardiograms and vital signs were not attributable to the study drug.

CONCLUSIONS

There was no demonstrable efficacy of AF0150-contrasted ECHO to evaluate for EBD, EF, SWM, or for myocardial perfusion in this patient population. The sponsor emphasized that the population selected for this protocol did not require suboptimal baseline ECHOs for enrollment.

C. TITLE – PROTOCOL # IMUS-003-USA

Safety, Dosing, and Efficacy Study of AF0150 in the Contrast-Ultrasound Assessment of Focal Lesions of the Liver or Kidney in Patients with CT-or MRI-Confirmed Abnormalities

STUDY DESIGN

A multicenter (all 6 centers were in the U.S.), open-label, 2-staged Phase 2 study investigating safety in such patients (see title); there is no placebo-control group.

Stage 1: The "pilot (purely safety) stage" enrolled 22 subjects; the 1st 6 patients were randomized to receive either a series of bolus doses (40 mg followed by a maximum of four 10- to 20-mg doses) or infusion. Afterwards (Amd. 3), each patient received an initial bolus followed by an infusion.

Stage 2: The "open (efficacy and safety) stage" enrolled 25 subjects, who each received a bolus dose of up to 1.0 mg/kg AF0150, followed by a titrated infusion of up to 160 mg AF0150.

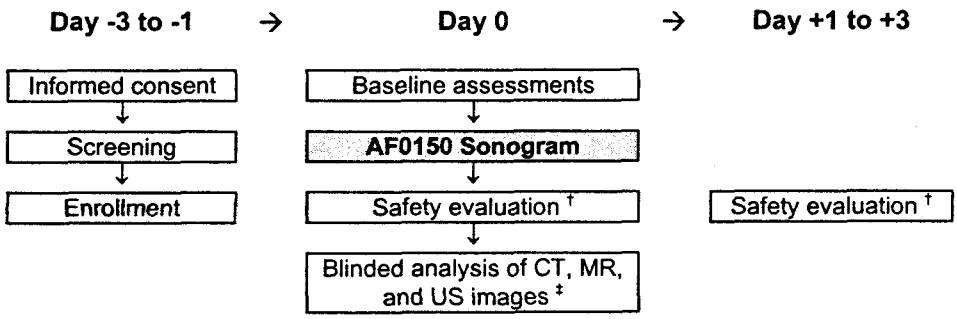
Doses used were based on contrast enhancement of the liver and kidney images in Protocol IMUS-001. However, the test dose used for this study (IMUS-003) is at a lesser concentration (10 mg/mL) rather than that used in IMUS-001 (20 mg/mL). Because of poorer sonographic visualization, Amendment #4 was established to increase the concentration back to 20 mg/mL. This was the concentration used in the "open stage of the study.

OBJECTIVES

1° objective: AF0150 safety and dosing strategy for patients with radiologically-confirmed focal lesions of the liver or kidney.

2° objective: efficacy using fundamental gray-scale, 2nd harmonic, and power and spectral Doppler ultrasound imaging.

STUDY FLOW CHART



† Safety evaluations were performed at baseline, at various times during th 1st and 4th hours post-AF0150, and Days 1, 2, and 3 post-AF0150. Subjects were to be followed for up to 14 days post-AF0150.

‡ To be conducted by an independent radiologist after enrollment is complete.

RESULTS

SUBJECTS ENROLLED

A total of 22 subjects were enrolled in the pilot phase of the study; 25 subjects were enrolled into the open phase.

Under Amendment 2 (the "original protocol"), 6 subjects were enrolled, with 4 randomized to a series of bolus doses (40 mg followed by a maximum of four 10- to 20-mg doses) and 2

randomized to infusion (80 mg infused). (Amendment #2 is called the "original protocol"; prior to that, the "original original" was issued, followed by Amendment #1 and then the FDA-requested changes were incorporated into Amendment #2.)

After the 1st 6 patients were enrolled, Amendment #3 was generated to primarily change the dosing regimen for each subject to receive up to 1.0 mg/kg AF0150, followed by a titrated infusion of up to 160 mg AF0150 (thus, the study is no longer randomized). Under Amendment 3, there were 16 subjects enrolled.

Because of poorer sonographic visualization with the AF0150 concentration at 10 mg/mL, Amendment #4 was established to increase the concentration to 20 mg/mL (the same concentration used in Protocol IMUS-001-USA). This was the concentration used in the "open stage of the study, where 25 subjects were enrolled to receive a bolus dose of up to 1.0 mg/kg AF0150, followed by a titrated infusion of up to 160 mg AF0150.

TREATMENT

Results from the randomized image reviews demonstrated that visualization of lesion vascularity was enhanced using AF0150, unlike non-contrasted sonography. Bolus AF0150 administration led to visualization in 10 (50%) of 20 subjects, versus 7 (33%) of 21 subjects who received infusional AF0150. In addition, there was an improvement in the ability to assess lesion vascularity in 11 (55%) of 20 subjects post-bolus AF0150, versus 8 (38%) of 21 subjects post-infusional AF0150. More diagnostic information could be obtained from fundamental gray-scale imaging post-bolus AF0150 (8 subjects) versus post-infusional AF0150 (2 subjects).

SAFETY

Please refer to the Integrated Safety Summary. Briefly, AF0150 was well tolerated in this study, with no deaths, serious or severe adverse events reported. Those AE's reported were mostly mild in severity. Of the 47 subjects enrolled, 25 (53%) subjects experienced at least one AE during the study, with a higher incidence of AEs reported on Day 0 (the day of dosing). The most commonly reported AE is headache (5 of 47 subjects; 11%), followed by chest pain, nausea (3 of 47 subjects each; 6% each); diarrhea and flatulence (2 of 47 subjects; 4%). Females (63%) were noted to have a higher incidence of AEs than males (48%), with the single AE noted with females being chest pain (3 females; 0 males). Severity of AE's was "mild" (reported in 17 subjects) and "moderate" (reported in 7 subjects); "unknown" severity was reported in 1 subject. No trends in individual changes from baseline laboratory values associated with AF0150 administration were observed; observed changes appeared to be related to subjects' underlying medical conditions.

CONCLUSIONS

Visualization of kidney and liver lesion vascularity with fundamental gray-scale imaging was noted with AF0150 administration. More diagnostic information was obtained from bolus than from the infusional imaging, particularly with regards to lesion borders. Thus, AF0150 was well tolerated at the specified dosage of 1 mg/kg bolus followed by 160 mg infusion dose.

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D. TITLE – PROTOCOL # IMUS-012-USA

An Open-label, Single-Dose Study to Assess the Pharmacokinetic Parameters and Rate of Elimination of Perfluorohexane after a 4-mg/kg Bolus Intravenous Injection of AF0150 in Healthy Adult Volunteers

STUDY DESIGN

A single-center (clinical site: [redacted]), single-dose, open-label, 2-staged Phase I study to evaluate the pharmacokinetics of **perfluorohexane (PFH = perflexane**, a perfluorinated alkane stabilizing gas which is diluted into N_2), an active component of AF0150.

Each subject received AF0150 at 4 mg/kg IV (= 20 μg PFH/kg) over 25 mL/min, followed by saline flush. This dose was selected to (1) include the highest dose tested in other clinical studies, and (2) to improve the ability to measure expired air and blood levels for drug analysis.

- **Pilot phase**: The purpose of this is "to test the logistics of the study procedures and verify all aspects of the sample collections and analyses". Plans to enroll 2 subjects.

- **Pivotal phase**: To enroll 10 subjects; pharmacokinetics, and blood & pulmonary clearances were studied.

Pharmacokinetic variables that were measured were as follows:

TABLE 12: IMUS-012-USA -- PHARMACOKINETIC DEFINITIONS

Parameter	Definition
$AUC_{0-\infty}$	Area under the curve from time 0 to infinity
AUC_{0-tqc}	Area under the curve from time 0 to the last quantifiable concentration time calculated by the trapezoidal rule
C_{max}	Maximum blood/air concentration
$T_{½}$	Elimination half-life of the drug in blood/air for PFH
Cl_{sys}	Total systemic clearance of PFH
Cl_{lung}	Lung clearance of PFH
MRT_{last}	Mean residence time from time 0 to last quantifiable time
$\%\ PFH_{0-3hr}$	Percent total recovery of PFH in expired air to 3 hours
$\%\ PFH_{0-\infty}$	Percent total recovery of PFH, extrapolated to 24 (48 hours for Subjects No. 109R, 110R, 112R and 113, with the suffix "R" signifying subjects who received a 2nd dose)
V_z	Apparent volume of distribution

ADDENDA

Amendment 3 was produced to clarify the interval between dosing in 3 subjects who received a 2nd dose of AF0150. Two exclusion criteria were modified to allow for previous AF0150 exposure (a washout period of at least 3 days) and recent participation in a clinical trial for those 3 patients.

RESULTS

SUBJECTS ENROLLED

Normal adult volunteers, 13 patients in total.

Subjects number 101 – 108, 111 and 113 received one IV dose, while subjects 109, 110, and 112 received 2 IV doses of 4.0 mg/kg (= 20 μg PFH/kg per dose) separated by a wash-out period of at least 25 days. Redosing was necessary due to technical difficulties suspected due to the marked differences noted in PFH expiration in those 3 subjects (Subjects 110, 111, and 112) when compared with the 1st 9 subjects (7 males; 2 females). An additional female subject, subject no. 113 was enrolled to receive one dose to rectify the technical difficulties.

DOSING

Bolus of AF0150 of 4.0 mg/kg (= 20 μg PFH/kg) infused manually at a rate ≤ 25 mL/minute, followed by a saline flush manually. An amendment was added to repeat dosing for 3 females who had discrepancies noted upon completion of the original test dose; a wash-out period of ≥ 3 days was added to accommodate these patients.

Pharmacokinetics

PFH was distributed to the lung after rapid delivery of the entire cardiac output to the lung; PFH then partitioned from the blood to the alveolar air. This activity appeared to follow first-order kinetics, with the PFH being virtually entirely eliminated by respiration and residual PFH in fat only, all of which was noted at the 24th hour. However, in Subjects 110, 111 and 112, all of which were female, the total recoveries of PFH was less (46%, 51%, and 46% respectively), when compared with the other females (Subjects 108, 109, and 113, with recoveries of 109%, 90%, and 87%, respectively). Technical difficulties are attributed by the sponsor to ill-fitting face masks; however, the sponsor does not rule out the possibility of a true pharmacokinetic difference.

Blood clearance appeared more variable than the clearance from expelled air (lung), with no statistical differences between male and female subjects in the rate and extent of PFH exposure in blood.

Below are tabulations of the pharmacokinetic data derived from this study (derived from tables provided by the sponsor in Volume 81 p 046); Table 13 compiles all patients' data, while Table 14 excludes data from Subject 111, who did not have a repeat dose. The parenthetical numbers in both tables represents the percent coefficient of variation (CV%); note that, when Subject 111 was excluded (in Table 12), the CV% associated with the AUC pharmacokinetic parameters for blood decreased by at least 4- to 5-fold. However, the CV% for the AUC parameters did not change for expired air when Subject 111 was excluded; thus, the terminal phase capatured by the concentration-time profile for this parameter in Subject 111 was in line with the other subjects.

TABLE 13: IMUS-012-USA – MEAN (CV%), n = 13

	$AUC_{0\text{-}24hr}$ (ng*hr/mL)	$AUC_{0\text{-}\infty}$ (ng*hr/mL)	T_{max}[a] (min)	C_{max} (ng/mL)	$t_{½}$ (hrs)	MRT_{last} (hrs)	Cl (L/hr)	% PFH (0-3 hr)	% PFH (0-∞)
BLOOD	8.8 (211.8)	11.3 (227.6)	2.0	26.3 (106.9)	5.7 (105.4)	3.4 (124.7)	662.9 (110.1)		
AIR	3.2 (23.8)	3.4 (20.0)	1.5	25.7 (42.5)	10.3 (64.3)	2.1 (81.3)	605.3 (14.9)	70.2 (32.9)	84.4 (24.9)

Source: Volume 81, pp 043 – 046 – Tables 11.4.2.1:1 – 2 and 11.4.2.2:1 – 2.

TABLE 14: IMUS-012-USA -- MEAN (CV%), n = 12

	$AUC_{0\text{-}24hr}$ (ng*hr/mL)	$AUC_{0\text{-}\infty}$ (ng*hr/mL)	T_{max}[a] (min)	C_{max} (ng/mL)	$t_{½}$ (hrs)	MRT_{last} (hrs)	Cl (L/hr)	% PFH (0-3 hr)	% PFH (0-∞)
BLOOD	3.7 (38.9)	4.2 (52.4)	2.0	28.0 (102.2)	5.3 (114.7)	2.7 (133.5)	716.3 (102.6)		
AIR	3.3 (21.2)	3.4 (20.9)	1.5	27.8 (29.7)	9.0 (55.1)	1.6 (32.3)	603.7 (15.6)	74.6 (23.6)	87.2 (22.1)

Source: Volume 81, pp 043 – 046 – Tables 11.4.2.1:1 – 2 and 11.4.2.2:1 – 2.

ADVERSE EVENTS

Clinical Adverse Events: Refer to the Integrated Safety Summary write-up. All subjects were included into the safety summary. There were no deaths or serious adverse events encountered in this study. Adverse events (AE's) were reported by 2 (15.4%) of the 13 subjects enrolled, with one incidence each of headache and pain (at the nose base). All AE's were mild, transient, and determined not to be related to the study medication.

TABLE 15: IMUS-012-USA -- ADVERSE EVENTS REPORTED (ALL SUBJECTS)

ID #	GENDER	AE	SEVERITY	RELATED	ONSET AFTER TX	DURATION
108	Female	Headache	Mild	Not Related	125 min.	2.3 hrs
110	Female	Soreness at the Nose Base	Mild	Not Related	45 min.	23.7 hrs

Source: Volume 081 p 059 (Table 12.2.1:1, modified).

Clinical Laboratory AE's: There were no changes in blood and urinalysis parameters from baseline significant enough to be designated as "Clinical Adverse Events".

Vital Signs: There were no changes in vital signs deemed significant enough to be called "Clinical Adverse Events".

Other Parameters: Other issues reviewed for adverse events, such as **neurological examination** (i.e., the mini-mental status examination changes), **cardiac monitoring** (i.e., telemetry, 12-ECG monitoring, and cardiac enzyme changes), and **arterial oxygen saturation**, did not have changes deemed significant enough to be called "Clinical Adverse Events".

CONCLUSIONS

PFH, after a 4-mg AF0150 intravenous bolus injection in healthy adult volunteers, distributed to the lung, as reflected by high exhaled-air levels within 1 minute after dosing. This is explained by the rapid delivery of the cardiac output to the lung and rapid release of PFH from blood to alveolar air. Disappearances of PFH appeared to follow 1st-order (linear) kinetics. Pharmacokinetic profiles from expired air appeared to be more complete than data obtained from blood following intravenous administration; lung clearance was more consistent than blood. No deaths or serious adverse events were reported during the study. In conclusion, the 4 mg/kg dose of AF0150 administered intravenously was safe and well-tolerated.

E. TITLE – PROTOCOL # IMUS-018-USA

An Open-Label Dose-Titration Study of 3 Doses of AF0150 in the Echocardiographic Assessment of Patients with Left Ventricular Dysfunction

STUDY DESIGN

A multicenter (both clinical sites were in California, U.S.A.), open-label, dose-ranging (multi-dose, with 3 doses per subject), non-randomized, non-controlled (no placebo), Phase 2 study.

The baseline echocardiograms consisted of 2 modes of imaging: (1) fundamental continuous and (2) fundamental gated imaging. Each of the 2 modes needed the following 3 different views: (1) apical 4-chamber, (2) apical 2-chamber, and (3) apical long axis. In order to obtain useful images, a minimum of 10 cardiac cycles were obtained in each view. The required sequence was as follows:

1. **Fundamental Continuous**:
   - Apical 4-chamber, apical 2-chamber, apical long axis: 10 cycles each.
2. **Fundamental Dual Gated*** – end-diastole and end-systole:
   - Apical 4-chamber, apical 2-chamber, apical long axis: 10 cycles each.

* Gating was done at the down-slope of the T-wave (end-diastole) and on the peak of the R-wave (end-systole), with triggers set simultaneously at a 1:1 interval.

After baseline imaging was performed, each subject was to receive 3 incremental doses of AF0150 in the following sequence: 0.125, 0.25, and 0.5 mg/kg, injected IV over @ 10 seconds with a 10-minute interval between each dose. Imaging, both fundamental continuous and gated, will follow each dose during each 10-minute interval. According to the sponsor, the continuous imaging modality allows for the greatest amount of microbubble destruction by ultrasound, and 10 minutes of such imaging allows for complete clearance of the drug (by microbubble destruction).

The sequence of 2-D echocardiographics will be as follows:

1. **Fundamental Continuous**:
   - Apical 4-chamber, apical 2-chamber, apical long axis: 10 cycles each.
2. **Fundamental Continuous**:
   - Apical 4-chamber, apical 2-chamber, apical long axis: 10 cycles each.
3. **Fundamental Dual Gated*** – end-diastole and end-systole:
   - Apical 4-chamber, apical 2-chamber, apical long axis: 10 cycles each.
4. **Fundamental Continuous**:
   - Apical 4-chamber, apical 2-chamber, apical long axis: 10 cycles each.
5. **Fundamental Continuous**:
   - Apical 4-chamber (performed for the remainder of the 10-minute imaging period following each dose to evaluate the duration of useful contrast enhancement).

* Gating was done at the down-slope of the T-wave (end-diastole) and on the peak of the R-wave (end-systole), with triggers set simultaneously at a 1:1 interval.

Efficacy was assessed in terms of the ability of AF0150 to opacify the left ventricle, which was divided into 3 regions – apical, middle, and basal. In each region, the opacification was graded by the blinded reviewer as: 0 = none; 1 = slight; 2 = moderate; and 3 = complete, or N = no view available for scoring. The mean opacification score was calculated by averaging the available scores from apical, middle, and basal portions of the left ventricular cavity.

Efficacy was based on videodensitometry measurements and blinded review of the echocardiographic images. Videodensitometry was performed for left ventricular opacification and duration of useful contrast enhancement. The reviewer was blinded to subject and dose. Mixed effects analysis of variance (ANOVA) was the statistical method used to analyze for quantitative (gated imaging of LV opacification by videodensitometry) and qualitative (blinded review) measures.

Safety was assessed on the basis of reported adverse events, clinical laboratory tests, vital signs, and EKG following treatment. Vital signs were assessed at baseline, 5 minutes after each dose, and at 1-hour and 24-hour follow-up periods. EKG's were monitored and recorded at baseline, at 30 minutes, and at the 1-hour and 24-hour follow-up periods.

Endpoints:

1. 1° endpoint (using both fundamental continuous and gated modes):
   a) LV opacification
      i) Mean score will be calculated by averaging the available scores from apical, middle and basal portions of the left ventricular cavity.

1. 2° endpoints (using only fundamental continuous mode):
   a) Duration of attenuation
   b) Duration of useful contrast enhancement
   c) Safety assessment.

ADDENDA

There was one Amendment (Amendment #1), which allowed for the following for this protocol, all prior to subject enrollment:

(1) Included a 2nd study site, to allow enrollment of up to 18 subjects and to record concomitant medications during screening of the subjects, and
(2) A multi-step, improved set of instructions for the constitution of AF0150 and standardized instructions for machine settings (in Volume 89 pp 116 – 118). Methods were not similar to the Phase 3 studies; also, as demonstrated on the CFR (Vol. 89, p 127-128), opacification was used to determine durations of attenuation and useful contrast enhancement.

Several changes to the planned analysis occurred; it is not mentioned as being a part of any Amendments. The changes are as follows:

(1) Because of a small range of ejection fraction values, analyses for effect of ejection fractions on left ventricular opacification, duration of opacification, and useful contrast enhancement was not explored.
(2) Analyses for linearity and quadratic effects were deemed unnecessary and thus not performed.
(3) Statistical analysis for left ventricular opacification was performed without a fixed effect for view in the mixed effects model.
(4) Tests for differences between all pair-wise least-square means were computed.
(5) The previous plans had no discussion regarding interpretation of "useful contrast enhancement duration" from the quantitative data. In order to determine "background noise", another study was used to evaluate the non-contrast images of 3 randomly chosen subjects. The cut-off score (a calculation of the baseline average pixel intensity for non-contrast images) was found later to be a score of 30, above which constituted the useful contrast enhancement. With this method, the differences in pixel intensity above the baseline noise could be determined between the 3 doses.
(6) Critical values for vital sign values for the safety analyses were included.

RESULTS

SUBJECTS ENROLLED

Adult volunteers (age range 18 – 80 years), 18 patients (13 were male) in total, were enrolled. Subjects with technically adequate echocardiogram demonstrating left ventricular ejection fractions in the range of 20% to 40% fit the inclusion criteria. All subjects had cardiac abnormalities reported; the most common abnormalities were

(1) congestive heart failure (14 subjects; 78%),
(2) unspecified essential hypertension (12 subjects; 67%),
(3) coronary atherosclerosis of unspecified type of vessel (native or graft) (8 subjects; 44%),
(4) undiagnosed cardiac murmurs (8 subjects each; 44%),
(5) other primary cardiomyopathies (7 subjects; 39%),
(6) hyperlipidemia (5 subjects; 28%),
(7) atrial fibrillation (5 subjects; 28%),
(8) unspecified adverse effect of medicinal and biological substance (5 subjects; 28%).

The number of subjects who received medications prior to and during the study remained the same for all but one medication – heparin, which was administered to 12 subjects (67%) on the day of dosing (but no patients received it before therapy). The most common medications used prior to enrollment were

(1) angiotensin-converting enzymes (ACE) inhibitors (plain) (14 subjects; 78%),
(2) digitalis glycosides (14 subjects; 78%),
(3) potassium (7 subjects; 39%),
(4) platelet aggregation inhibitors excluding heparin (6 subjects; 33%),
(5) vitamin K antagonists (5 subjects; 28%),
(6) angiotensin II antagonists (plain) (5 subjects; 28%),
(7) hydroxymethyl glutaryl coenzyme A (HMG CoA) reductase inhibitors (5 subjects; 28%),
(8) organic nitrates (5 subjects; 28%).

Protocol deviations/violations --

(1) Entry Criteria -- One patient, Subject #01-003, was enrolled despite a violation of an entry criterion (i.e. 18 – 80 years); the patient was 87 years old.
(2) AF0150 Dosing – One patient, Subject #02-002, received half the protocol-specified dose at each dose level because of an error in recording the subject's weight. Those doses were 0.064, 0.127, 0.252 mg/kg.
(3) Echocardiographic Imaging – Four patients, all at Site 01 (Subjects –003, –004, –005, and –006), did not have the 10-minute continuous imaging sequence after the 0.5-mg/kg dose. Instead, pulse-inversion harmonic imaging and 2nd harmonic imaging were used for these patients. Additionally, Subject 01-006 did not have the apical long axis view imaged after each dose was administered.
(4) Missing Safety Assessments – Missing assessments are tabulated below; of note, safety parameters were assessed at time points specified in the protocol for some patients. In addition, 5 blood chemistry parameters (alkaline phosphatase, albumin, cholesterol, triglycerides, and glucose) were not listed on the Laboratory Test Case Report Form and thus were not collected.

TABLE 16: IMUS-018 – LIST OF ENROLLED SUBJECTS WITH MISSING ASSESSMENTS

ASSESSMENT	SUBJECT	MISSING ASSESSMENT
Hematology	01-006	All parameters at 1 hour.
Chemistries	02-004	Fibrinogen at 1 hour.
	01-001	Total protein at 24 hours.
	01-002	All parameters at baseline; total protein at 1 and 24 hours; CK and CK-MB at 1 hour.
	01-003	Baseline CK and CK-MB
	01-004	Total and direct bilirubin, and CK and CK-MB at 1 hour; total protein at 24 hours.
	01-005	Total and direct bilirubin at 1 hour.
	02-002	CK-MB at 24 hours
	All subjects	Alkaline phosphatase, albumin, cholesterol, triglycerides, and glucose
Urinalysis	01-001	All parameters at baseline, 1 hour, and 24 hours.

Source: Volume 088 p 031 (Table II).

EFFICACY (TREATMENT)

I. DEMOGRAPHICS

See "SUBJECTS ENROLLED" section above ; 17 of the 18 total subjects received AF0150 as per protocol.

II. TREATMENT COMPLIANCE

All 18 patients received 3 incremental doses of AF0150 with a 10-minute interval between each dose during which continuous imaging was used to allow complete clearance of the drug by maximizing bubble destruction. There did not appear to be any carry-over effects from previous doses for efficacy assessment at each dose level. No subject had a combined duration of attenuation and duration of useful contrast enhancement of over 9 minutes.

III. ANALYSIS

Videodensitometry was performed [redacted] for left ventricular opacification and duration of useful contrast enhancement.

TABLE 17: IMUS-018-USA – EFFICACY RESULTS (CUMULATIVE)

EFFICACY PARAMETER	ECHOCARDIOGRAPH (2-D CONTRAST)	APICAL 4-CHAMBER	APICAL 2-CHAMBER	APICAL LONG AXIS
LV OPACIFICATION				
• Quantitative	Gated mode (Videodensitometry)	Not significant.	P = 0.0007 0.125 vs 0.5	P = 0.0003 0.125 vs 0.5
• Qualitative	Continuous mode	Not significant.	Not significant.	Not significant.
	Gated mode	Not significant.	P = 0.0013 0.125 vs 0.5	Not significant.
DURATION OF USEFUL CONTRAST ENHANCEMENT				
• Quantitative	Continuous mode (Videodensitometry)	Not significant.	Not significant.	Not significant.
• Qualitative	Continuous mode	P = 0.0013 0.125 vs 0.25	P = 0.0001 0.125 vs 0.5	Not significant.
DURATION OF ATTENUATION				
	Continuous mode	P = 0.0001 0.125 vs 0.25	P = 0.001 0.125 vs 0.5	P = 0.001 0.25 vs 0.5

IV. Duration of Attenuation (DOA) and Duration of Contrast Enhancement (DUCE)

The DOA was tested in IMUS-018, where DOA was defined as complete attenuation of any portion of the left ventricle, which would include the myocardium or cavity. As tabulated below, the mean duration of attenuation at the proposed clinical dose (0.125 mg/kg) was 0.43 minutes (25.8 seconds). This has increased significantly with each dose level, with 0.25 mg/kg at 1.0 minute (60 seconds) and with 0.5 mg/kg at 1.57 minutes (94.2 seconds).

TABLE 18: IMUS-018-USA – Duration of Attenuation Data

Duration of Attenuation Data	AF0150 Dose 0.125 mg/kg (N = 18)	0.25 mg/kg (N = 18)	0.5 mg/kg (N = 18)	Dose (mg/kg) Comparison* (P value)
Number of Subjects	N = 18	N = 18	N = 18	0.125 vs 0.25 (0.0001)
Mean ± SD (minutes)	0.43 ± 0.35	1.00 ± 0.68	1.57 ± 0.79	0.125 vs 0.5 (0.0001)
Range (minutes)	0.00 – 1.30	0.00 – 2.20	0.40 – 3.30	0.25 vs 0.5 (0.0001)

Source: Volume 44 p 136; Table VII.43
* ANOVA; confidence level = 99%

The DUCE was also tested in IMUS-018, where DUCE was defined as the duration in which there is moderate intensity of contrast throughout the cardiac cycle independent of where it occurred within the ventricular cavity. As tabulated below, the mean duration of contrast enhancement at the proposed clinical dose (0.125 mg/kg) was 1.22 minutes (73.2 seconds). This has increased significantly with each dose level, with 0.25 mg/kg at 2.22 minute (133.2 seconds) and with 0.5 mg/kg at 2.93 minutes (175.8 seconds).

TABLE 19: IMUS-018-USA – Duration of Contrast Enhancement Data

Duration of Contrast Enhancement Data	AF0150 Dose 0.125 mg/kg (N = 18)	0.25 mg/kg (N = 18)	0.5 mg/kg (N = 18)	Dose (mg/kg) Comparison* (P value)
Qualitative Assessment				
Number of Subjects	N = 18	N = 18	N = 18	0.125 vs 0.25 (0.0013)
Mean ± SD (minutes)	1.22 ± 0.92	2.22 ± 1.30	2.93 ± 1.65	0.125 vs 0.5 (0.0001)
Range (minutes)	0.00 – 3.10	0.00 – 4.40	0.10 – 6.20	0.25 vs 0.5 (Not listed)
Quantitative Assessment				
Number of Subjects	N = 18	N = 18	N = 18	No statistically significant differences quantitatively.
Mean ± SD (minutes)	1.28 ± 1.53	2.50 ± 2.77	2.83 ± 2.53	
Range (minutes)	0.00 – 4.00	0.00 – 8.00	0.00 – 9.00	

Source: Volume 44 p 137; Table VII.45
* ANOVA; confidence level = 99%

Safety [Adverse Events (AE's)]
All subjects were included into the safety summary. There were no deaths or serious adverse events encountered in this study; all other adverse events were assessed as mild to moderate in intensity, with none considered serious. AE's reported consisted of one event each of moderate hypokalemia and increased lactic dehydrogenase. Both of these events were considered by the investigator as possibly related to study drug and resolved without treatment.

Conclusion

This was a Phase 2 dose-ranging study to assess AF0150 administered in 3 doses (0.125, 0.25, and 0.5 mg/kg). All 18 subjects received 3 incremental doses of AF0150; 17 subjects were dosed

according to protocol and 1 subject received half the dose at each dose level. A dose-dependent increase in left ventricular opacification was noted for both the quantitative (videodensitometry) and qualitative (blinded view) assessments. Statistically significant increases in left ventricular opacification were observed at each dose for the quantitative assessment, as well as for the qualitative assessment in gated mode for mid and high doses compared to low doses. However, with the use of the standard clinical method of continuous mode, no statistically significant difference was noted. There was a dose-dependent increase in duration of attenuation and duration of useful contrast, suggesting a higher AF0150 dose for patients with EF ≤ 40% if a longer duration for contrast imaging is needed. There were no deaths or serious adverse events encountered in this study; all other adverse events were assessed as mild to moderate in intensity, with none considered serious. The dose of 0.125 mg/mL AF0150 was sufficient to opacify the left ventricle in patients with LV dysfunction (i.e., EF 20% to 40%); there was little improvement derived from using higher doses. There were no baseline echo results for within-patient comparison.

APPEARS THIS WAY
ON ORIGINAL

VI. KEY EFFICACY STUDIES

TITLE

PROTOCOL # IMUS-007-USA

A Multicenter, Saline-Controlled Study of AF0150 in the Echocardiographic Assessment of Left Ventricular Function in Patients with Suboptimal Noncontrast Images

PROTOCOL # IMUS-008-USA

A Multicenter, Open-Label Study of AF0150 in the Echocardiographic Assessment of Left Ventricular Function in Patients with Suboptimal Noncontrast Images ✳

✳ This pivotal study, supportive of IMUS-007-USA, is of similar design but has unique areas which are *italicized and double-underlined*, to be distinguished from the IMUS-007-USA.

STUDY DESIGN

- Multicenter (all 18 centers were in the U.S.); *for IMUS-008-USA, 11 centers in the U.S., all different from IMUS-007-USA*
- Single-blind; *IMUS-008-USA was open-label.*
- Blinded image read, using 3 independent echocardiologists to evaluate for endocardial border delineation (EBD), ejection fraction (EF), and segmental wall motion (SWM)
- Placebo- (saline-) controlled (for safety only); *for IMUS-008-USA, there was no placebo-controlled arm.*
- Single-dose
- Randomized for the 1st 160 patients for safety evaluation; *not for IMUS-008-USA, in which all patients received AF0150*
- Images are randomized and pre- and post-AF0150 and are read independently

Subjects were to be enrolled based upon echocardiographic criteria (the "qualifying echocardiogram") mentioned in the Inclusion Criteria section. Within 72 hours prior to the day of dosing, a screening examination was performed, which would include a "confirmatory echocardiogram" which would re-confirm eligibility (see Amendment #2). For IMUS-007, the 1st 160 subjects enrolled were to be randomized to either the saline control (n = 80) or AF0150 (n = 80). Randomization was in a 1:1 ratio at each site with a block size of 4. The next 130 subjects entered into the study were assigned to receive AF0150. Subjects were randomized to 1 of 2 groups according to a schedule generated by the [redacted] Within 1 hour of dosing, a resting, baseline non-contrast 2-D echocardiogram (n-ECHO) using fundamental, continuous imaging is to be performed. Three views will be collected, in the following order: (1) apical 4-chamber, (2) apical 2-

chamber, and (3) apical long axis. A minimum of 10 cardiac cycles will be obtained in each view and recorded on S-VHS videotape.

STUDY OBJECTIVES

The overall objective of this study was to demonstrate the visual efficacy of AF0150 when used as a contrast agent in echocardiography with at least one of the 1° endpoints:

(1) effects of AF0150 on endocardial border delineation (EBD) when compared with baseline non-contrast echo, and

(2) impact upon ejection fraction (EF); when compared with baseline non-contrast echo to determine which has closer agreement with the "truth" standard test – radionuclide ventriculography (RVG).

1° OBJECTIVES –

Ability of AF0150 to improve the assessment of cardiac function, as measured by endocardial border delineation (EBD), and ejection fraction (EF) of the left ventricle based on echocardiograms in the fundamental continuous mode in patients undergoing resting 2-D gray-scale echocardiography. Improved assessment was determined by comparing:

- Baseline non-contrast with contrast EBD; and
- Baseline non-contrast EF with contrast EF to determine which had better agreement with gated equilibrium RVG, a gold standard for EF measurement.

2° OBJECTIVES –

1. Evaluate if AF0150 improves the echocardiographic assessment of cardiac function (EBD & EF) using fundamental gated mode in patients undergoing resting 2D gray-scale echocardiography.
2. Evaluate SWM as measured by baseline non-contrast (n-ECHO) and contrast (c-ECHO) echocardiogram in the fundamental continuous mode; *for IMUS-008-USA, the SWM as measured by both n-ECHO and C-ECHO was evaluated versus SWM measured by gated magnetic resonance imaging (MRI).*
3. Evaluate the safety of AF0150.

ENTRY CRITERIA

INCLUSION CRITERIA

1. Adults (≥ 18 years of age) of either gender (non-pregnant; non-lactating) with a clinical diagnosis requiring echocardiography, leading to a sub-optimal* non-contrast echocardiogram.

   * There must be failure to visualize 2 to 9 segments in the apical 4- and 2-chamber views of the baseline echocardiogram. Also there must be ≥ 1 segment visualized in both views for an estimate of EF to be made. This qualifying echocardiogram must be confirmed on the day of dosing before obtaining the baseline non-contrast echocardiogram. See "Protocol Amendment" section regarding the reason for the addition of the "confirmatory" echocardiography, due to either missing segments in the "qualifying" echocardiograms, and/or use of HP5500 machines leading to inconsistent settings in determining whether the echocardiographic readings are eligible.

2. Patients must have a normal sinus rhythm; occasional (≤ 6 / minute) ectopic beats are permitted provided there is no interference with interpretation of the echocardiogram.
3. Patients must have a gated RVG performed within 48 hours (before or after) of AF0150 or saline administration.
4. *For IMUS-008-USA, patients must have a gated MRI performed within 48 hours (before or after) of AF0150 at 2 specific study sites.*

Exclusion Criteria

1. Patients with clinically unstable conditions within 24 hours of planned AF0150 or saline administration (*no saline administration in IMUS-008-USA*).
2. Patients with ejection fractions < 20%, suspected cardiac shunt or moderate to severe valvular disease.
3. Participation in a clinical trial involving an investigational drug (including AF0150 therapy) or device within either the preceding 4 weeks or the period of ≤ 7 half-lives of the study drug, whichever is longer, or participation in > 4 clinical trials within the past year.

STUDY PLAN

Dosing and Administration

For all subjects enrolled, study drug (or placebo) was administered within 1 hour following the baseline non-contrast echocardiogram (n-ECHO).

An intravenous catheter [redacted] [redacted]) and extension tubing [redacted]) were used for administration of AF0150 or saline. AF0150 is prepared

by constitution of 200 mg dry powder with 10 mL sterile water for injection (SWFI) to a final concentration of 20 mg/mL. Patients assigned to AF0150 received AF0150 IV bolus injection of 0.125 mg powder/kg body weight (0.00625 mL/kg) over 10 seconds, followed by a 3-mL saline flush over 10 seconds to ensure complete delivery of the study drug. Within the appendices of the submission, the dose of AF0150 in milliliters range from 0.25 mL (for a person weighing 40 kg) up to 0.75 mL (for a person weighing 119 kg). For patients randomized to the saline group, the dosage is also calculated by the product of patient's weight (0.00625 mL/kg).

Reconstitution of AF0150 with 10 cc SWFI; the AF0150 solution, after agitating well, should have an opaque-white appearance. A 1.0 mL syringe will be used to withdraw an amount of the AF0150 greater than the calculated amount needed in the study. This is to allow the investigator to displace any large bubbles in the syringe; the extra volume will also be discarded, with the calculated dose remaining within the syringe.

The Angiocath™ and Small Bore Extension Set™ are a low-resistance intravenous catheter system recommended for this study. Immediately before AF0150 administration, 3.0 mL of 0.9% NaCl will be adminstered as a flush. Afterwards, the AF0150 is to be administered over 10 seconds; the pressure applied during administration (not mentioned in quantitative terms) must not lead to clearing of the solution (damage toward the microbubbles).

The dosage chosen was based upon Protocol # IMUS-001-USA, involving normal volunteers, where the dose was the lowest dose that showed contrast enhancement of echocardiograms. Another protocol, Protocol # IMUS-018-USA validated the dosage, which sufficiently opacified the left ventricular cavity in fundamental continuous and gated imaging modes in patients with left ventricular dysfunction (i.e. EF 20% to 40%). (Little improvement in opacification was derived from using higher AF0150 doses other than an extended contrast image duration time.)

Imaging Procedure

Immediately following dosing, the contrast echocardiogram was to be obtained in the following order: (1) apical 4-chamber view, (2) apical 2-chamber view, and (3) apical long axis view. A minimum of 10 cardiac cycles will be obtained in each view, in order to eliminate bias in selection of images, and will be recorded on S-VHS videotape. This sequence should be repeated for at least 2 minutes following any attenuation. Attenuation of the images may occur for up to 30 – 60 seconds post-AF0150 injection. The required images must be obtained after attenuation has subsided and while contrast appears dispersed from the apex to the base. If attenuation is not apparent then the required images should be obtained without delay, repeating the sequence for at least 2 minutes. The investigator must record on the case report form (CRF) the following: (1) the time that each view is captured on the videotape, (2) the duration of attenuation following each dose, and (3) the duration of useful imaging, defined as the time during which contrast-enhanced images can be obtained.

The echocardiogram device settings will **remain unchanged** from pre- and post-contrast ECHOs. Those baseline settings necessary to maintain the mechanical index (transmit power) will be at the lowest setting to minimize bubble destruction, as listed below.

Mechanical Index	Range of 0.4 to 1.0
Gain	Optimized to discretion
TGC	Optimized to discretion
Dynamic Range	60 – 70 dB: 150dB for the ATL 5000
Compression	70 – 80 dB
Persistence	Off
Post Processing	Linear (is A; is Gray Map 1)
Single Focal Zone	Atrial or basal zone

The ultrasound scanner has a higher mechanical index (MI) as the default setting (MI – 1.7; earlier scanners have MI's at 1.0 to 1.1); therefore there was premature ultrasonic destruction of the AF0150 microbubbles occurring before completion of image acquisition. (There was attenuation followed by rapid loss of contrast with . Therefore, the protocol was amended to exclude the use of this machine.

The sequence of 2-D echocardiographics will be as follows:

1. **Fundamental Continuous**:
   - Apical 4-chamber, apical 2-chamber, apical long axis: 10 cycles each.
2. **Fundamental Continuous**:
   - Apical 4-chamber, apical 2-chamber, apical long axis: 10 cycles each.
3. **Fundamental Dual Gated** – end-diastole and end-systole:
   - Apical 4-chamber, apical 2-chamber, apical long axis: 10 cycles each.
4. **Fundamental Continuous**:
   - Apical 4-chamber, apical 2-chamber, apical long axis: 10 cycles each.

Gated radioventriculography † [RVG; a.k.a. multi-gated acquisition (MUGA) scan] must be performed within 1 to 48 hours prior to or following AF0150 dosing to provide a standard of truth for evaluation of EF in all patients. Stress gated RVG scans are not acceptable for this study. *For IMUS-008-USA, the MRI ‡ is to be performed within 1 – 48 hours prior to or following AF0150, in this case to serve as a standard of comparison for evaluation of SWM.*

† The RVG data will be collected from several hundred cardiac cycles to generate an image set of the beating heart that is presented as a single, composite cardiac cycle. A modified *in vitro* labeling method (Ultratag®, Mallinckrodt) will be used. Acquisition parameters should be the following:

#Frames per R-R interval	Minimum of 16 to 32 frames
Count density	Minimum of 200 K per frame
Matrix	64 x 64
Percent acceptance window	20%

A blinded, trained technologist will calculate the EF's from all available data, using . One (1) independent blinded reviewer, a Nuclear cardiologist, is selected by Alliance to review all regions of interest (ROIs) obtained during the ejection fraction calculation from the RVG (see below). This independent reviewer verifies the correct ROI description of the EBD.

‡ *For IMUS-008-USA, regarding MRI reading, an independent cardiologist with expertise in MRI reading, will complete the image analysis of SWM from the MRI images. This individual was not to be involved in any other analysis of study images (i.e. echocardiogram or RVG). The settings will be as follows:*

Short-axis scan (Multi-phase, multi-slice)	
Field of view	30-35 cm
Matrix	*Readout*: 256 *Phase-encoding direction*: 8 151 *K-space segmentation*: Used as long as time per frame < 40ms (e.g. for a minimum TR = 13, group size at 3 = time per frame: 39 ms)
Slice thickness	8 mm
Slice gap	2 mm

Tagged 4C, Tagged 2C, Tagged LAX	
FOV	35 cm
Matrix	256 x 256
Avoid K-space segmentation	
Temporal resolution	< 30 ms
Slice thickness	8 mm
Slice gap	2 mm
Tags	15 mm apart

Core Laboratory Evaluation (Tape Handling):

The core laboratory for imaging [redacted] will receive all ultrasound and RVG data including tapes, digital media, and representative film copies. The core laboratory will identify and digitally capture ultrasound segments from the S-VHS tape using site-specified times from the transmittal form, on which each site will identify the times of the required apical 4-chamber, apical 2-chamber, and apical long axis segments for the pre- and post-dose segments. The core laboratory will review the imaging data for each of the required views to determine the images with the best endocardial border delineation, which will be saved to the appropriate patient folder. These data will serve as the data for blinded review; 18 individual movies will be made per subject.

[redacted] will prepare the digital/video data for evaluation of EBD, SWM and EF. I [2] will also capture 4 still frames determined by the "selector" to be used in the calculation of each ECHO EF. (One each for the 4-chamber diastolic, 4-chamber systolic, 2-chamber diastolic, and 2-chamber systolic.) The "selector" is an independent, blinded, Echo cardiologist selected by Alliance and who is not an employee of Alliance, who will review all ECHO image data to identify an end-diastolic and end-systolic frame from a single cardiac cycle for each view. The selector will be blinded to study treatment and will not be involved in any other blinded read of any other study images (i.e. RVG or MRI).

The image specialist† will draw the Region of Interest (ROI) for the End Diastolic (ED) and End Systolic (ES) views on the 4-chamber and 2-chamber views. All ECHO ejection fractions (EF) will be calculated (using the Bi-Planar Method) by the protocol-specific image specialist (employed by I^2) using the pre-identified image frames. Each patient will have 6 ejection fraction calculations performed.

† This was a post-hoc change in the protocol; refer to comment #1a. under "Critique of Design" section.

Analysis Of Efficacy: Blinded Read Protocol

Three independent "blinded" readers will evaluate each patient's images (apical 4-chamber, apical 2-chamber and apical long axis views) obtained from non-contrast echocardiography (n-ECHO) and contrast echocardiography (c-ECHO). The readers will assess continuous loops of baseline and contrast images of apical 4- and 2-chamber views displayed simultaneously with selected still frames. Endocardial border delineation (EBD → 1° endpoint), segmental wall motion (SWM → 2 endpoint) and ejection fraction (EF → 2° endpoint) will be evaluated sequentially. RVG is the standard method ("gold standard") for evaluation of EF.

Three independent, board-certified echocardiologists will review images for all patients. Prior to the blinded review, a "training" session in the use of ultrasound contrast agents for rating EBD will be conducted by another independent echocardiologist † to standardize the process.

† This was a post-hoc change in the protocol; refer to comment #1b. under "Critique of Design" section.

Pre- and post-contrast images will be displayed independently in a randomized fashion. The 3 blinded reviewers will each either agree or disagree with the ROI by the core lab. If there is a disagreement, the blinded reader will redraw the ROI, and the EF will be recalculated. The images available will be the best images available during the entire dosing sequence. For the ejection fraction, the "bi-planar disc" method will be used, images calibrated, and a curvilinear line will be fit to manually-placed points which demarcate the endocardial borders. The readers will review data in 3 stages, when the images have been processed for 30%, 70% and 100% of patients.

A total of **16 evaluable segments** will be obtained, consisting of

(a) **6** segments from the **apical 4-camber** view (each segment representing the following cardiac walls: basal septal, mid septal, apical septal, apical lateral, mid lateral, and basal lateral)

(b) **6** segments from the **apical 2-chamber** view (each segment representing the following cardiac walls: basal inferior, mid inferior, apical inferior, apical anterior, mid anterior, and basal anterior), and

(c) **4** segments from the **apical long axis** view (each segment representing the following cardiac walls: basal posterior, mid posterior, mid anterior septal, and basal anterior septal).

TABLE 20: NUMBERED ECHOCARDIOGRAM SEGMENTS

ECHO VIEW	SEGMENT #	CARDIAC WALL
Apical 4-chamber	Segment 1	Basal septal
	Segment 2	Mid septal
	Segment 3	Apical septal
	Segment 4	Apical lateral
	Segment 5	Mid lateral
	Segment 6	Basal lateral
Apical 2-chamber	Segment 7	Basal inferior
	Segment 8	Mid inferior
	Segment 9	Apical inferior
	Segment 10	Apical anterior
	Segment 11	Mid anterior
	Segment 12	Basal anterior
Apical long axis	Segment 13	Basal posterior
	Segment 14	Mid posterior
	Segment 15	Mid anterior septal
	Segment 16	Basal anterior septal

Endocardial Border Delineation

The readers will read the n-ECHO and c-ECHO images and score each of the 16 segments (listed below); efficacy is gained if statistical significance is seen for 2 out of the 3 readers.

0 = no delineation;
1 = mild or fair delineation (inadequate to assess function);
2 = moderate or good delineation (adequate to assess function);
3 = excellent delineation (excellent demarcation of borders throughout the cardiac cycle);
N = no view available for segment.

The following calculations will be used to evaluate the endocardial border delineation (EBD).

1. **Total EBD score** (possible score range: 0 to 48)
   a) = sum of the scores from each of the 16 segments for that patient
2. **EBD change** (within-patient) **score** (possible score range: -48 to +48)
   a) = Post-contrast ECHO total EBD score – baseline (pre-contrast) ECHO total EBD score
3. **Linear model** † to analyze **EBD Change** using analysis of variance (**ANOVA**) methods
   a) EBD change = (overall mean change) + investigational site + experimental error
   b) Weight least squares (WLS) method: Used if ANOVA method is inadequate
   c) Hypothesis for the 1° endpoint
      i) **Null** = overall mean is 0 (no statistical difference between post- & pre-contrast ECHO total EBD scores)
      ii) **Alternate** = overall mean is NOT 0; if + , then efficacy has been demonstrated

† This was a post-hoc change in the protocol; refer to comment #1c. under "Critique of Design" section.

For each patient, the total number of segments (sum of the number of the 16 segments visualized) for n-ECHO and c-ECHO images will be calculated. A "within-patient visualization change score" will be calculated (= number of visualized segments for c-ECHO minus number of visualized segments for n-ECHO). In the original submission, the overall mean change and variability due to reader, investigators, and readers-by-investigators interaction will be analyzed using analysis of variance procedures with the following general linear model:

Change score = (overall mean score) + reader + investigator + reader * investigator

The sponsor hypothesizes that the c-ECHO will have a significantly different (2-tailed alternative hypothesis) mean number of visualized segments when compared to n-ECHO. The echocardiogram assessment with AF0150 contrast (when compared to non-contrast evaluation) must have a positive overall mean change (least-squares estimate) and the p-value must be ≤ 0.05.

Additional analyses performed to support the efficacy evaluation of AF0150 upon EBD include

(1) a **"By-Segment"** analysis, in which each segment is analyzed using a WLS method (same linear method as described above);
(2) a **"By-Site"** analysis, if there is a significant difference among investigational sites is noted in the "By-Segment" analysis;
(3) a **"By-View"** analysis, in which total EBD scores by-view (pre-contrast, post-contrast, and change scores) will be analyzed; and
(4) a **"By-Site"** analysis, if there is a significant difference among investigational sites is noted in the "By-View" analysis.

Finally, **"scenarios"** were established in case of subjects having missing data for individual segments. For a **"no-change scenario"** in which the baseline n-ECHO or c-ECHO value, but ***not both***, was missing for an individual segment, the missing value was replaced by the non-missing value. If ***both*** were missing, then both were set to 0 (no delineation). For a **"worst-case scenario"** in which the value for an individual segment was missing, then the missing baseline n-ECHO value was set to 3 (excellent delineation) and the missing c-ECHO value was set to 0 (no delineation).

Ejection Fraction

The EF will be categorized as either

1) > 65%,
2) 55 – 65%,
3) 45 – 54%,
4) 35 – 44%,
5) 25 – 34%, and
6) < 25%.

The categories chosen are based upon a modified version of left ventricular dysfunction system devised by Maseri (Vol. 44, pp 26 – 27), in which a 10% interval for EF risk-stratification in patients surviving myocardial infarction. The effect of AF0150 upon the visualization of EF will be assessed by evaluating the change in EF from the baseline (n-ECHO) to the post-contrast (c-ECHO) images when both are compared to RVG. Two contingency tables† (4-by-4 dimension) will be made by cross-classifying the EF classes:

Table 1: n-ECHO (Baseline pre-contrast) vs. RVG; and
Table 2: c-ECHO (Post-contrast) vs. RVG.

† **This was a post-hoc change in the protocol; refer to comment #1d. under "Critique of Design" section.**

A weighted Kappa statistic will be used to evaluate the strength of the agreement within Tables 1 and 2. Afterwards, a two-tail binomial test for paired samples will be applied to calculate the exact probability for determining if the proportions between categories are significantly different than 0.5 (see the Statistical Review). In this particular case, patients will be assigned to one of the following 4 categories:

1. Baseline pre-contrast ECHO & RVG agree, AND post-contrast ECHO & RVG agree.
2. Baseline pre-contrast ECHO & RVG ***dis***agree, AND post-contrast ECHO & RVG agree.
3. Baseline pre-contrast ECHO & RVG agree, AND post-contrast ECHO & RVG ***dis***agree.
4. Baseline pre-contrast ECHO & RVG ***dis***agree, AND post-contrast ECHO & RVG ***dis***agree.

There is agreement if ECHO and RVG EF results are assigned the same EF classes. There is disagreement if ECHO and RVG EF results are assigned different EF classes.

Hypothesis for the 1° endpoint regarding the ejection fraction (EF):

1. **Null** hypothesis = % agreement for post-contrast ECHO (when compared to RVG) ***is not*** significantly different from the % agreement for the pre-contrast baseline ECHO (when compared to RVG).
2. **Alternative** hypothesis = % agreement for post-contrast ECHO (when compared to RVG) ***is*** significantly different from the % agreement for the pre-contrast baseline ECHO (when compared to RVG).
    a. **Efficacy is demonstrated** (the null hypothesis rejected) if post-contrast ECHO agreement with RVG ***is greater than*** the baseline pre-contrast ECHO agreement with RVG.
    b. For the **binomial test** (equivalent to the McNemar's test), only categories **2 and 3** are necessary for discriminating efficacy information.
    c. AF0150 contrast is significantly more effective than without contrast, when each is compared to RVG (the standard of truth) **if (1)** the proportions between categories 2 and 3 are significantly different than 0.5*, ***and*** **(2)** if the proportion in category 2 is greater than the proportion in category 3.

        * The "0.5" value is the expected value if there is no difference in agreement between baseline n-ECHO (when compared with RVG) and post-contrast c-ECHO (when compared with RVG).

A p-value ≤ 0.05 will be considered statistically significant. If the McNemar's methods are not adequate for assessing for differences among investigational sites, a weighted least squares (WLS) method will be used for categorical data.

Finally, to account for subjects with missing values, **"scenarios"** were established. For the **"no-change scenario"**, if EF data were missing for ***either*** the baseline n-ECHO ***or*** C-ECHO and the RVG data were available, the missing EF value was set to be the same as the non-missing value. If EF data were missing from ***both*** the baseline n-ECHO and c-ECHO and a RVG value was available, data were set to the category of "baseline n-ECHO and RVG agree". Subjects missing RVG data were excluded from this analysis. For the **"worst-case scenario"**, if the baseline n-ECHO data were missing, then the value was set to "agree with RVG".

Segmental Wall Motion †

The blinded readers will read the n-ECHO and c-ECHO images and assign one of the following scores to each of the 16 segments, as tabulated below. An electronic case report form (CRF) was programmed to accept a particular SWM score based upon the EBD score. Therefore, as tabulated below, if the subject receives an EBD score of 0 or 1, the electronic CRF will record an SWM score only of 0. A subject with an EBD score of 2 or 3 will lead to an SWM score greater than 0.

TABLE 21: PHASE 3 PROTOCOLS – SEGMENTAL WALL MOTION DEFINITIONS

SWM Definition	SWM Score	EBD Score	EBD Definition
Segment not visualized	0	0 or 1	No or mild-to-fair delineation (inadequate to assess function)
Normal	1	2 to 3	Moderate-to-good or excellent delineation (able to assess function)
Hypokinesis	2		
Akinesis	3		
Dyskinesis	4		
Aneurysmal	5		
No view available for segment	N	N	No view for the segment

"Extent-of-agreement" (EOA) data, defined as the number of the 16 segments that 2 readers for a particular reader-pair have with the same SWM assessment score, will be calculated. To determine if differences in the EOA between n-ECHO and c-ECHO images, a "Within-patient" EOA score will be used; this is c-ECHO EOA minus n-ECHO EOA. Patient listings of the EOA change scores will be presented by the 3 reader-pairs. The overall mean change and variability due to reader-pairs, investigators and reader-pairs by investigators interaction will be evaluated using analysis of variance procedures. The sponsor speculates the efficacy of AF0150 will be demonstrated; in this case, the mean value would be positive.

For the subgroup of subjects in IMUS-008-USA who had a MRI, each segment was to be assigned to the categories above except "N". For these subjects, the % segments assigned to the same functional category on both baseline n-ECHO and MRI was to be calculated. Also, the % segments assigned to the same category on both c-ECHO and MRI was calculated. For some subjects, both untagged and tagged MRIs were available and evaluated for SWM. If a segment from an untagged MRI was assessed as "N", then the assessment of the tagged MRI for the same segment was used in the analysis. Otherwise, assessments of untagged MRIs were used.*

* *Tagged MRI studies are MRI studies that are computer-generated upon a grid system and used for wall thickness and contours of the heart (therefore for anatomic rather than functional purposes). Untagged MRI studies were used to evaluate wall motion (function).*

† **Most of this was post-hoc for the protocol; refer to comment #1e. under "Critique of Design" section.**

Subset analyses were to also be performed, as per the request of the Agency. The categories and description of the subsets are tabulated below. These apply to all 3 variables (EBD, EF, SWM).

TABLE 22: PHASE 3 STUDIES – CATEGORIES EVALUATED FOR THE SUBSET ANALYSES

Categories	Subsets
Gender	Male / Female vs. Study Population
Race	Caucasian vs. Non-Caucasian vs. Study Population
Age	< 65 years / ≥ 65 years and ≤ 80 years / > 80 years
Body Surface Area	≤ 2 m^2 / > 2 m^2 vs study population
Concomitant Medications	Reported in ≥ 20% of subjects in both studies combined vs study population
Diagnosis	Reported in ≥ 20% of subjects in both studies combined vs study population
Cardiac Disease (RVG EF)	EF < 50% / EF ≥ 50% vs study population
COPD and other Conditions	All subjects with these conditions / subjects without these conditions vs study population

Supporting Efficacy Data (from Volume 44, pp 134 – 137)

1. Duration of Attenuation (DOA)

Duration of attenuation was defined as the time from the 1st appearance of the contrast bolus ("blacking out" the image due to excessive signal back-scatter in the apical 4-chamber view) to the time when the attenuation subsided to the level of the mitral valve. The DOA was to be tested in both IMUS-007 and IMUS-008. The DOA was also tested in IMUS-018, where DOA was defined as complete attenuation of any portion of the left ventricle, which would include the myocardium or cavity.

2. Duration of Useful Contrast Enhancement (DUCE)

Duration of useful contrast enhancement was defined as the time that the contrast provided clinically useful enhancement of the endocardial borders without the effect of attenuation. The DUCE is a somewhat subjective test in both IMUS-007 and IMUS-008. The DUCE was also tested in IMUS-018, where DUCE was defined as the duration in which there is moderate intensity of contrast throughout the cardiac cycle independent of where it occurred within the ventricular cavity.

STUDY MONITORING

Below is a tabulation of the monitoring plans. Adverse events (AE's) were monitored throughout the study and coded using the Coding Symbols for a Thesaurus of Adverse Reaction Terms (COSTART) dictionary.

TABLE 23: PHASE 3 PROTOCOLS – STUDY MONITORING

TEST / PROCEDURE	SCREENING	DOSING		FOLLOW-UP				
	- 72 hour	Pre	Post	5 min	15 min	30 min	1 hour	24 hour
Hx / Phys. Exam	X							
Mini-Mental Status Exam (MMSE) * ‡	X					X		X
Baseline ECHO		X						
Laboratory Studies								
Serum β-hCG	X							
Hematology		X					X	X
Coagulation Tests		X					X	X
Chemistries		X					X	X
Urinalysis		X					X	X
Clinical (Unit) Monitoring								
Vital Signs		X		X	X	X	X	X
12-lead ECG		X		X			X	X
Pulse Oximetry*		X		X	X	X	X	X
Cardiac Studies								
n-ECHO		X						
c-ECHO			X					
Gated RVG*		X	X					
Gated MRI *		X	X					

* Pulse oximetry (and also mental status evaluation) conducted in the 1st 120 patients enrolled.

‡ MMSE tests for **orientation** (2 questions; max. score: 10); **registration** (1 question; max. score: 3); **attention/calculation** (1 question; max. score: 5); **recall** (1 question; max. score 3); and **language** (6 questions; max. score: 9) → **total max. score** = 30.

* *For IMUS-008-USA, the SWM as measured by gated magnetic resonance imaging (MRI) is to be conducted within 48 hours of the c-ECHO..*

[redacted] is the central clinical laboratory where blood and urine is sent for analysis.

Normal ranges for all laboratory parameters were submitted into Section 8.XIII.C, Listing 11 (Volume 74, pp 326 - 340). An additional change to the planned protocol included documenting those events that have no clinical manifestation but potentially can lead to a clinical adverse event, labeled as "potentially clinically significant" changes or levels (defined below). Although the sponsor states (in the Table of Contents and within the text) that the values for "potentially clinically significant" levels are located in Section 8.XIII.C, Listing 12 (Volume 74, pp 341 - 423), ***no section can be found within the submission***.

Potentially Clinically Significant Laboratory Adverse Events

The "potentially clinically significant (PCS)" value is defined (Volume 44 p194) as either a "panic-alert" or a "telephone-alert" value. The **"telephone-alert"** value is defined to highlight moderately low or moderately high values to the attention of the investigator for appropriate follow-up. The **"panic-alert"** value is considered more critical than the "telephone-alert" value; the "panic-alert"

value is a value suggestive of potentially serious or adverse conditions. There is no section that can be found that illustrates the PCS values of any laboratory study. Specific definitions are as follows:

- Potentially clinically significant laboratory abnormalities† – a normal, abnormal or missing baseline value that shifted to a "panic" value. If a "panic " range was not provided by the central laboratory for a specific laboratory assessment, the telephone range (range to notify investigator) was used to as a guide for determining potentially clinically significant abnormalities.

- Potentially clinically significant vital sign changes – the ranges are listed in Volume 90 p 45.

- Potentially clinically significant oxygen saturation change ($Sa0_2$) – any change > 5% from baseline and an $Sa0_2 \leq 95\%$.

- Potentially clinically significant MMSE change from baseline, to a > 3-point decrease from the baseline value.

- Potentially clinically significant ECG changes were also mentioned to be evaluated (Volume 90 pp 45 – 46).

† **Most of this was post-hoc for the protocol; refer to comment #1f. under "Critique of Design" section.**

APPEARS THIS WAY
ON ORIGINAL

PROTOCOL AMENDMENTS

Below is a tabulated summary of the essential changes of the protocol. Essential changes in the protocol were:

(1) inclusion of both fundamental continuous and fundamental gated imaging in the efficacy evaluation in Amendment 1;

(2) the addition of a confirmatory screening echocardiogram recommended for Day 0 (pre-dosing) in Amendment 2; and

(3) exclusion of patients from the efficacy analysis who had echocardiograms acquired on the [redacted] machine.

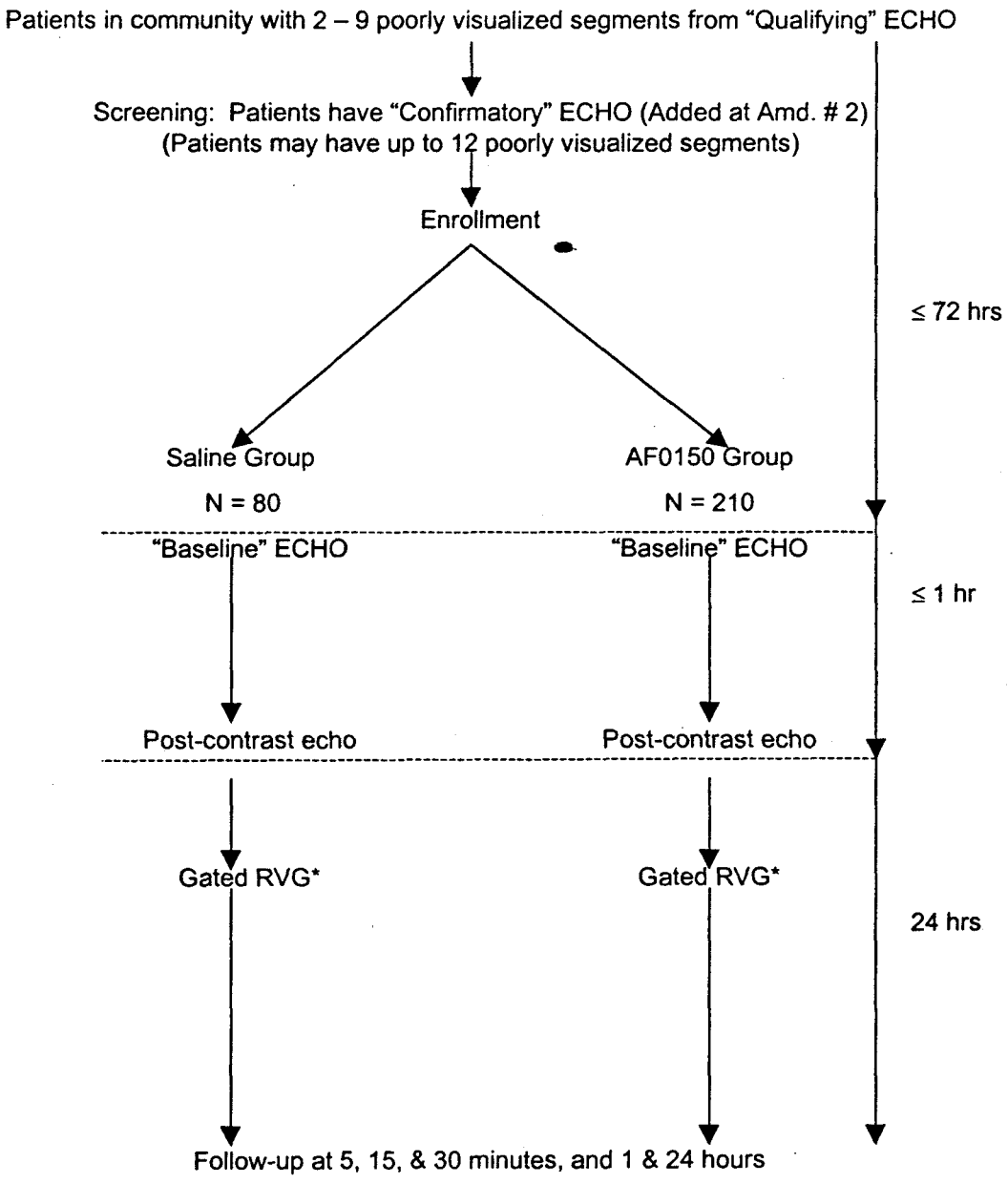
TABLE 24: PHASE 3 PROTOCOLS – PROTOCOL AMENDMENTS

Protocol Amendment & Date	Summary of Essential Changes
Amendment 1 (13 March 1998)	• Changed the evaluation of EF from a 2° to a 1° efficacy endpoint. • Increased sample size from 220 to 280 subjects. The 1st 160 subjects were to be randomized in a 1:1 ratio to either saline or AF0150 and the next 120 subjects were to be assigned to receive AF0150. • Modified the definition of sub-optimal echocardiogram. • Included both fundamental continuous and fundamental gated modes of imaging in efficacy evaluation. • Clarified and modified the timing of safety assessments.
Amendment 2 (10 July 1998)	• Increased sample size (IMUS-007: from 280 to 305 subjects; IMUS-008: from 200 to 250 subjects). Redefined the Efficacy Population to include only those subjects enrolled after Amendment 2. • Clarified that 1° efficacy analyses were to be based on continuous mode imaging. • Provided recommended machine settings for imaging. • Provided additional guidance on reconstitution and administration of saline and AF0150.
Amendment 3 (14 December 1998)	• Adjusted sample size (IMUS-007: from 305 to 290 subjects; IMUS-008: from 250 to 230 subjects). • Redefined the Efficacy Population for efficacy to include subjects enrolled before Amendment 2 whose images were not taken with [redacted].
Addendum 1* (13 January 1999)	• Defined the methodology for the evaluation of SWM from the images obtained by MRI • Removal of the determination of EF by MRI.

* Changes reflected in the Addendum are noted in the following locations:
  - IMUS-007-USA – Appendices L (Volume 93, p 093-207) and M (Volume 110, p 110-213)
  - IMUS-008-USA – Appendices L (Volume 171, p 171-207) and M (Volume 188, p 188-213)

IMUS-007-USA Study Flow Chart:

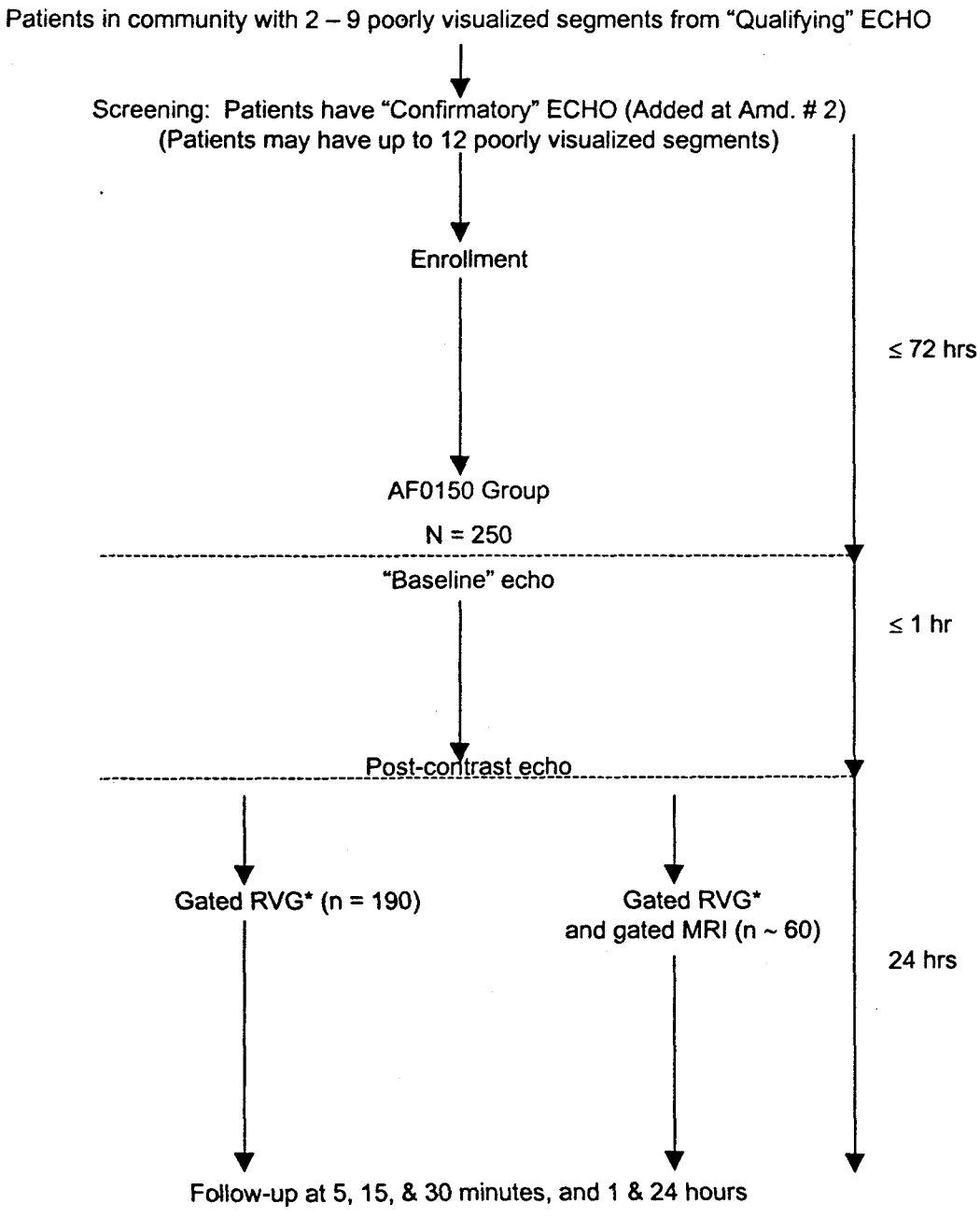
From Appendix in Vol. 93, p 093-184 (Section 16.1, page 1270), modified by clinical reviewer.



* May be conducted 1 to 48 hours before or after baseline echo.

IMUS-008-USA Study Flow Chart:

From Appendix in Vol. 109, p 109-316 (Section 16.1, page 974)



* May be conducted 1 to 48 hours before or after baseline echo.

CRITIQUE OF DESIGN

1. There are several post-hoc issues appearing that relate to tape handling:
   a) The original protocol had blinded readers drawing the regions of interest (ROI). The study report has the specialist drawing the ROI which are then confirmed by the blinded reader.
   b) The independent echocardiologist who trained the blinded readers is also a blinded selector for Study IMUS-008.
   c) Analyses for the SWM that were post-hoc included the use of an electronic case report form and the "extent-of-agreement" data.
   d) No "panic" value or "potentially clinically significant" reference ranges could be found in the submission; the correct location (if present in the submission) needs to be provided.
2. Patients with > 9 segments poorly visualized were excluded during the screening; excluding this patient population may have a deleterious impact on demonstrating efficacy of Imavist™.
3. EBD TOTAL score (or the sum score) was used; a segment analysis is needed.
4. EBD was not done at end-diastolic or end-systolic; nevertheless, EF was reviewed at ED and ES, so a correlation is needed.
5. Telemetry was needed during the study.
6. There are no present standards for EBD for ultrasound bubble contrast agents.
7. Regarding the purpose of the core laboratory, the blinded readers should have been allowed to review the whole continuous echocardiograms instead of the best images as chosen by the core laboratory, as well as determine the ejection fractions without the help of the core laboratory.

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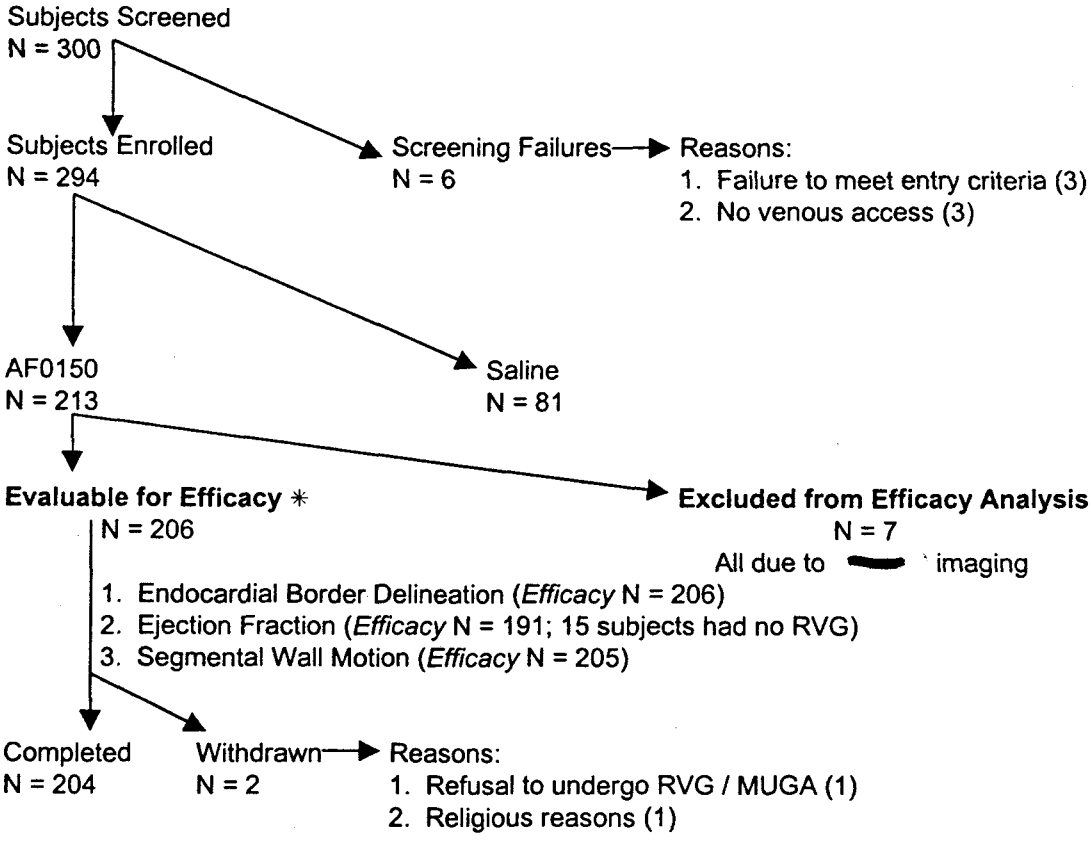
ON ORIGINAL

EFFICACY RESULTS

Disposition of Enrollees

For Protocol IMUS-007-USA, 294 patients were enrolled into 15 investigational sites (total sites = 18); 81 patients were randomized into the saline group and 213 patients received AF0150. Of the saline-treated group, 79 (98%) subjects completed the study; in the AF0150 group, 211 (99%) completed the study. The 4 patients enrolled into the study that did not complete the study had not discontinued due to adverse events.

Protocol IMUS-007-USA



* The **"evaluable for efficacy" population**: Defined as subjects who received AF0150 either after Amendment 2 or prior to Amendment 2 who were imaged with equipment other than the [redacted]. Within this population is the actual **"efficacy"** population for each variable (EBD, EF, and SWM), which is the population that fulfilled all requirements necessary to analyze the respective variable.

For the AF0150 group in IMUS-007-USA, out of the 213 patients who received AF0150, there were 206 (97%) *evaluable* for efficacy for all 3 parameters – EBD, EF, and SWM. All 206 patients were listed in the "efficacy" population for EBD. Because 15 patients had no RVG performed, there were 191 patients within the "efficacy" population for EF. Finally, 205 patients were listed within the "efficacy" population for SWM. Although 2 subjects in the saline group are also listed in

the table below, the saline group was not studied for efficacy and are thus assessed for safety purposes only.

TABLE 25: PROTOCOL IMUS-007 – PROTOCOL VIOLATIONS AND WITHDRAWALS

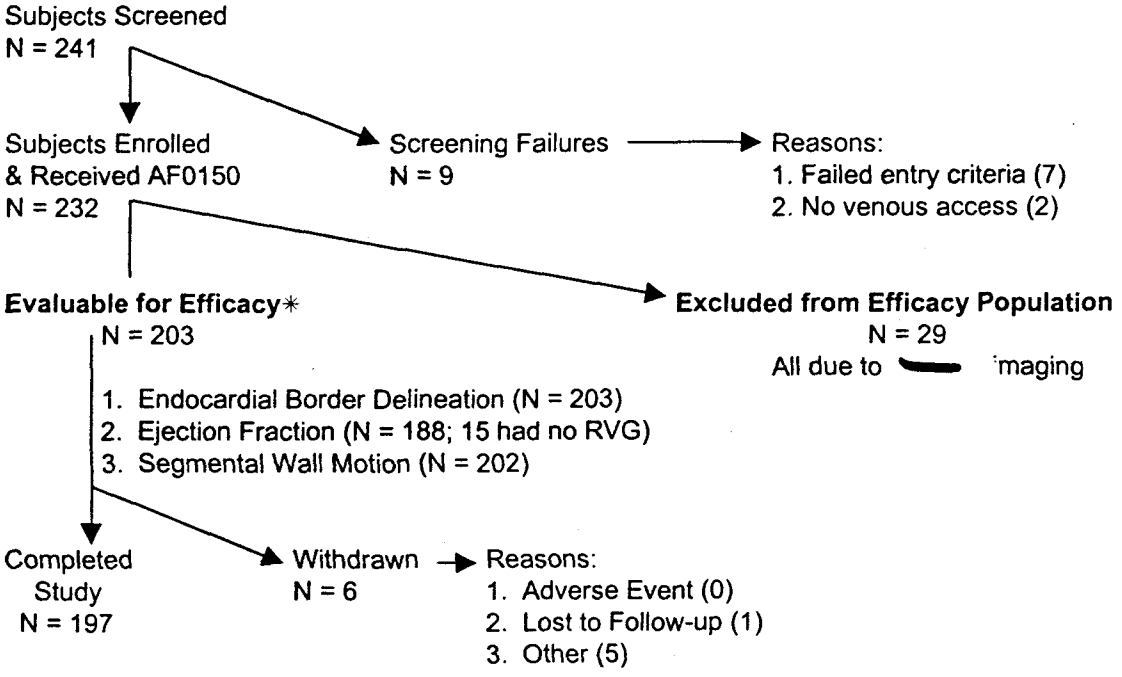
	Pt No.	Reason Not Completed Protocol IMUS-007-USA (Vol 95, p168)
Protocol Viol.	5-997	Confirmatory echo not suboptimal
	5-998	Screen failure
	5-999	Screen failure
	7-999	Exclusion criteria
	8-998	Unable to obtain IV access
	8-999	Withdrew consent (husband did not want patient to participate)
	10-998	Unable to obtain IV access
	10-999	Unable to obtain IV access
	11-005	Premature Ventricular Contractions > 6 / minutes
	11-999	Premature Ventricular Contractions > 6 / minutes
AF0150 withdrawal	5-035	Religious beliefs
	8-010	Cold room, hard bed; patient refused to cooperate for MUGA/RVG

All subjects in the AF0150 group with protocol deviations were included in the efficacy analysis; the following deviations were noted:

- 8 had entry criteria deviations, all of whom had > 6 ectopic beats/minute
- 29 had the following imaging violations (imputed using the "no-change" and "worst-case scenarios" sections):
  - 8 subjects → AF0150 had improper gating;
  - 2 subjects → AF0150 had missing images; and
  - 10 subjects → AF0150 had inadequate imaging where either the minimum number of cycles was not imaged or an incomplete/inappropriate view was obtained.

For IMUS-008, out of the total of 232 patients enrolled who received AF0150, there were 203 (97%) patients evaluable for efficacy (for all 3 parameters – EBD, EF, and SWM). The other 29 patients that had received AF0150 were not evaluable due to being imaged prior to Amendment 2. All 203 patients were in the "efficacy" population for EBD; however, 188 of those patients were in the "efficacy" population for EF due to 15 subjects having had no RVGs. Regarding SWM evaluations for efficacy, 202 patients were in the efficacy population.

Protocol IMUS-008



* The **"evaluable for efficacy" population**: Defined as subjects who received AF0150 either after Amendment 2 or prior to Amendment 2 who were imaged with equipment other than the [redacted] Within this population is the actual **"efficacy"** population for each variable (EBD, EF, and SWM), which is the population that fulfilled all requirements necessary to analyze the respective variable.

Tabulated below are 2 groups of subjects in IMUS-008. Fourteen (14) of the subjects were "protocol violations" and therefore excluded from the "evaluable for efficacy" population. Two of the subjects listed under "Protocol Violations" had been treated prior to being determined to be ineligible ("protocol violation"). Six of the 203 subjects evaluable for efficacy withdrew from the study and these subjects are also tabulated below.

TABLE 26: PROTOCOL IMUS-008 – PROTOCOL VIOLATIONS AND WITHDRAWALS

	Pt No.	Reason Not Completed Protocol IMUS-008-USA (Vol 107, p168)
Protocol Viol.	20-999	Unable to obtain IV access.
	22-009	Unable to obtain IV access.
	23-030	No IV access; no blood obtained.
	23-036	Not in sinus rhythm.
	23-037	Unstable condition → hypertensive crises
	28-006	Inclusion # 5 = No ***(Received 0.12367 mL/kg)***
	28-999	Alliance Corp. requested patient not to be dosed
	30-010	Atrial fibrillation with pacemaker
	30-011	Too technically difficult
	30-015	Multiple ectopic beats
	30-026	Inclusion # 5 = No ***(Received 0.12598 mL/kg)***
	30-097	Patient did not meet exclusion criteria
	30-098	More than 6 ectopic beats per minute
	30-099	Pacer in atrial fibrillation
AF0150 withdrawal	23-014	Traffic accident and work schedule.
	23-025	IV failure; no contrast seen.
	24-024	RVG cancelled (per Alliance Corp.); no comparison w/ harmonic
	25-004	Lost to follow-up
	27-024	RVG not done due to scanner malfunction
	27-033	RVG scanner out of service; test not done

ANALYSIS OF EFFICACY

Primary efficacy endpoints were visual effects of AF0150 on fundamental continuous mode when evaluating for (1) endocardial border delineation and (2) ejection fraction. The ability to determine efficacy for the latter endpoint (EF) is based upon (1) improved visualization of the former endpoint (EBD) and (2) greater agreement with RVG (the "truth" standard for evaluating EF) when compared with baseline non-contrast echo. At least one of the 1° endpoints must demonstrate efficacy to meet the study objective.

Screening (Qualifying and Confirmatory) vs. Baseline (n-ECHO) Echocardiograms

Below is a table illustrating the percentages of non-visualized segments upon screening for both qualifying and confirmatory echocardiograms. Those segments which were noted to be most consistently non-visualized were segments of the lateral cardiac wall (viewing the apical 4-chamber's segments 4 – 6) and the anterior cardiac wall (apical 2-chamber's segments 10 – 12). Note that the screening echocardiograms do not have the apical long axis view included.